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TRANSDOMINANT REV AND PROTEASE MUTANT PROTEINS OF HIV/SIV AS POTENTIAL ANTIVIRAL AGENTS IN VITRO AND IN VIVO

ANNUAL REPORT

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13. ABSTRACT *(Maximum 200 words)*

The major goal of this contract is to use gene therapy to target essential genes of HIV/SIV in order to inhibit virus expression. Transdominant mutants of the rev and protease genes were to be constructed, assayed for their in vitro inhibitory activity, optimized for expression in appropriate retrovirus vectors, and evaluated in an in vivo model, i.e., the SCID-Hu-PBL system. We have so far constructed mutants of HIV-1 rev and HIV-2 protease, and demonstrated that one of the rev mutants indeed has a transdominant phenotype. Our plan for year two will be to continue these studies, to develop in parallel the infection of SCID mice using molecularly cloned HIV-1 and HIV-2 under study, and to incorporate antisense expression vectors directed at the RRE to inhibit Rev activity.

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RA 1; HIV/SIV; AIDS; Transdominant mutants; Rev; Protease; Retrovirus vector; SCID-Hu Mice models

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FOREWORD

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PI Signature Thomas Wayne Strand Date 10-26-91

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INTRODUCTION:

AIDS continues to be a grave global epidemic associated with a sexually transmitted and blood borne pathogen, namely HIV. The latest statistics from epidemiological studies indicate that young adults (male and female) are among the major risk groups for infection. Therefore, the disease will have a tremendous impact on the workforce of all segments of society, including the military. Although a lot of progress has been made in the last few years relating to the molecular dissection of the HIV genome, as well as to efforts for vaccine and therapy development, there is as yet no "magic bullet" in sight. With regard to therapy, although there are some compounds already in the clinic that show some efficacy, most notably the deoxynucleoside analogs targeting the virus reverse transcriptase, the toxicity of these drugs as well as the emergence of drug resistant virus mutants significantly limit their long-term use. Therefore it is important to expand as much as possible our arsenal of anti-viral drugs to use in combination regimens. Such combinations may be particularly effective if they target different phases of the virus life cycle, i.e., the events prior to and after the integration event.

The genome of HIV encodes at least nine genes. Three of these, *gag, pol* and *env* are common to all retroviruses and engender either proteins that form the constituents of the virion particles (*gag* and *env*), or enzymes that are essential for virus replication (*pol,* which yields reverse transcriptase/RNase H, protease and integrase). Of the six remaining accessory genes of HIV, only two have been found to be essential for virus replication, namely, the *tat* and *rev* genes. Tat is a positive transactivator that presumably acts at both transcriptional and post-transcriptional levels to enhance virus expression and is required for expression of all viral proteins, while *rev* differentially activates expression of viral structural proteins post-transcriptionally (see Vaishnav and Wong-Staal, 1991 for review). Since both *tat* and *rev* are essential regulatory genes, they are, along with the viral enzymes, suitable targets for anti-viral strategies. The *rev* gene is a particularly attractive target because of our previous finding that an incomplete inhibition of *rev* could nonetheless result in complete block of Gag/Pol protein expression (Matsukura, et al., 1989), suggesting that virus expression is extremely sensitive to rev depletion.

Rev is a 20 kD phosphoprotein predominantly localized in the nucleoli (Cullen, et al., 1988; Felber, et al., 1989). It promotes the synthesis of viral structural proteins by increasing the level of unspliced (Gag-Pol) or singly spliced (Env, Vif) mRNA in the cytoplasm (Feinberg, et al., 1986). Although the precise mode of action of Rev has not been elucidated, it clearly represents a novel mechanism of gene regulation that affects the equilibrium between splicing and nuclear export of mRNA. Site-directed mutagenesis has revealed two functionally important domains in the protein: a nuclear/nucleolar localization domain which also contains a consensus RNA binding sequence, and a potential transactivating domain (Malim, et al., 1989). Genetic alterations in the transactivating domain has been shown to generate transdominant mutants.

In the present contract, we propose to use gene therapy for delivery of anti-viral compounds. The latter assume the forms of transdominant mutants of two key viral proteins: the protease enzyme and Rev. Although at the time we submitted this proposal, gene therapy was still largely an untrodden path, the ground has now been broken as the first patients are being treated by this novel approach. We are excited and optimistic that gene therapy will play an important role in controlling HIV infection in the near future.

Our objectives are as follows:

1. To construct mutants of *rev* and *protease* genes using site-directed mutagenesis of rev cDNA clones of HIV-1 and SIV_{mac} and *protease* genes derived from infectious clones of HIV-1 and HIV-2.

2. To evaluate the capacity of these mutants to inhibit activity of corresponding wild-type proteins in co-transfection experiments.

3. To insert these transdominant mutants in an amphotropic murine retrovirus vector and optimize expression in human T-cells using different internal promoters.

4. To transduce human PBL cells or neoplastic T-cell lines with retrovirus expressing these transdominant mutants to determine if uninfected cells could be protected from de nove infection or chronically infected cells would be repressed in virus production.

5. To determine if SCID-Hu mice reconstituted with human PBL transducing viruses carrying the transdominant mutant genes would be refractory to HIV infection and/or virus-induced CD4 cell depletion.

SCIENTIFIC PROGRESS:

Our strategy for accomplishing the specific goals is outlined in Fig. 1. The first phase of the project then was to construct the appropriate wild-type mutant genes. We first subcloned the coding regions of the *rev* and *protease* genes by PCR amplification of the pertinent regions of the respective wild-type viruses (see Table I) using appropriate 5' and 3' synthetic oligonucleotide primers (see Table 2). Each PCR fragment was then cloned into two vectors: (1) pSVL, an efficient eukaryotic expression vector for in vitro functional analysis of the genes in eukaryotic cells such as Cos-1, and (2) M13mp19, a single-stranded DNA bacteriophage that will enable us to perform site-directed mutagenesis using synthetic oligonucleotides. The general cloning strategy is depicted in Fig. 2. Each clone was verified by nucleotide sequencing for their authenticity.

The next step was to construct site-specific mutants of the four wild-type genes using the two-primer method of oligonucleotide-directed mutagenesis (Sambrook, J., Fritsch, E.F., and Maniatis, T. 1987). As a start, we chose a few sites in each gene, and began altering them by mutagenesis. The choice of these sites is based on previous findings that amino acid changes in them are both inactivating, and trans-dominant (Malim, et al., 1989). The proposed set of mutations are shown in Table 3 and Fig. 3. All the synthetic oligonucleotides used as mutagenic primers in these constructions were synthesized and purified in our laboratory (See Table 4).

Thus far, we have three confirmed mutations by dideoxy sequencing in the HIV-1 *rev* gene: 1.4, 1.3, and 1.5 and one confirmed mutation in the HIV-2 protease gene: 4.2. The last HIV-1 *rev* gene mutation is a fortuitous one which has been isolated together with mutant 1.4 (refer to Table 3). We have subcloned three mutant genes in the eukaryotic expression vector, pSVL and expressed each of the proteins, as well as their wild-type counterparts, in Cos-1 cells. We performed Western blot experiments on the cell lysates from these transfection experiments using polyclonal rabbit anti-Rev antibody. Results indicate that the relevant proteins are actually being produced, as evidenced by a band at ca. 17-18 kdal (data not shown). Functional assays of the Rev mutants are described below.

A. p1401

The structures of the three Rev variants are depicted in Figure 3A. p1401 has a $F_{38}F_{39}$ mutation in the polyarginine tract of Rev, the domain of the protein thought to be responsible for its RNA binding and nucleolar localization properties. This segment is also thought to be part of the multimerization domain, which is responsible for the protein's ability to bind to and multimerize on its RNA target, the Rev-Response Element (RRE) (Malim et al., 1991). One report proposed that the Rev protein may ultimately "coat" the entire viral RNA, a process which may be important in Rev function (Wingfield, P., et al., 1991). Mutation in this region is predicted to affect any of these above functions and is expected to yield an inactive protein. However, such a negative phenotype is expected to be recessive.

B. p1440

This variant *rev* has a double amino acid mutation $E_{10}D_{11}$---- $L_{10}Y_{11}$, near the N-terminus of the protein. Since this alteration is outside of the two critical domains of *rev*, (namely, the nucleolar localization/ multimerization domain at 35-50 and the trans-acting domain at 78-80), it is expected to possess wild-type phenotype.

C. p1.3

This plasmid encodes a *rev* gene containing a L_{78}---- N_{78} mutation in the *trans*-acting domain, spanning positions 78-80. This domain is thought to interact with as yet unknown cellular factor(s) and hence, is thought to be crucial in the functional mechanism of *rev* action. As such, this *rev* variant is expected to be inactive. In addition, since this protein has intact multimerization domain, it would be able to heteromultimers with the wild-type protein and interfere with the activity of the latter. Alternatively, it could also act as a competitive inhibitor for binding to the RRE target. As such, it is expected to have a *trans*-dominant phenotype.

We have carried out functional characterization of the three *rev* mutant genes along with wild-type *rev* as a control. The plasmid pIIIAR (gift of C. Rosen) which consists of LTR-CAT-RRE sequences is dependent on both tat and rev functions for CAT expression. As shown in Fig. 4, the rev mutants

with alterations in the leucine-rich domain (p1403 or 1.3) and arginine rich domain (p1401 or 0.1) are inactive when transfected in Cos-1 cells and did not yield CAT activity over and above that seen by tat transfection alone, while a rev mutant with alterations in the amino-terminal sequences (p1404 or 0.4) is functionally equivalent to wild-type rev. Furthermore, co-transfection of wild-type rev and mutant 1.3 revealed that the latter exerted a negative dominant phenotype such that at a ratio of 2:1 mutant:wt DNA, almost complete inhibition was observed. These results are consistent with what has been reported previously, and defined the leucine-rich domain to be the activator domain (Malim, et al., 1989; Mermer, et al., 1990).

The transdominant property of the 1.3 mutant was further assessed in Hela cells constitutively producing Rev (Hela-Rev). In this system, although considerable CAT activity was detected upon tat transfection alone (Fig. 5, lane 1), a supply of exogenous wildtype rev DNA by transfection further increased activity of the pIIIAR plasmid (Fig. 5, lane 6). Transfection of increasing amount of 1.3 DNA (1 to 6 μg) led to increasing reduction of CAT activity in a dose dependent manner.

The HIV-2 Protease Variants

The mutant HIV-2 protease we have available has a change in T_{26} ---- V_{26} (fig. 3B). The phenotype of this mutant is not yet determined. We are currently exploring a simpler method to assay the HIV protease *in vitro*, using an octapeptide substrate containing the consensus tyr--procleavage site with covalently attached fluorescence probes in the N- and C-terminus.

CONCLUSIONS:

Because of our relocation to a new laboratory at the beginning of the year, progress was somewhat slow. Nonetheless, we were able to generate mutant constructs of rev and protease. Specifically, one of the rev mutants was shown to have a transdominant effect *in vitro.* Thus, we are ready to proceed to the second phase of the project, which is to optimize the level of expression of these mutant genes in appropriate retrovirus vectors. Our plans for year two, in addition to pursuing these experiments, would also include: (1) Setting up an infection model in SCID-Hu PBL mice, using molecularly cloned viruses of HIV-1 and HIV-2 currently in use in this laboratory. (2) To diversify the gene therapy approach to include transdominant mutants of other HIV genes as well as other ways of targeting the Rev transactivation pathway. For example, we have very encouraging data using anti-sense oligonucleotides directed at the Rev Response Element (RRE) for virus inhibition (Wong-Staal, 1991). Our plan would be to express the corresponding anti-sense sequence in a retrovirus vector. Our decision to broaden our scope is based on observations of other investigators (G. Pavlakis; B. Cullen; personal communications) that transdominant Rev mutants are variable in their effectiveness for inhibiting virus.

TABLE 1.

SUBCLONING BY PCR:

	GENE TO CLONE:	LENGTH OF PROTEIN PRODUCT: (# a.a.)	SOURCE TEMPLATE PLASMID	PCR - AMPLIFIED REGION: (nt. positions)	SOURCE OF TEMPLATE PLASMID:
1.	HIV-1 rev	116	pKK rev	--------	J. Rusche, Repligen
2.	SIV_{mac} rev	107	p8	339-662	S. Colombini, LTCB
3.	HIV-1 protease	99	pHXB2gpt	2252-2548	R. Sadaie, LTCB
4.	HIV-2 protease	99	B12	2079-2375	G. Franchini, LTCB

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aa = amino acids
nt = nucleotide

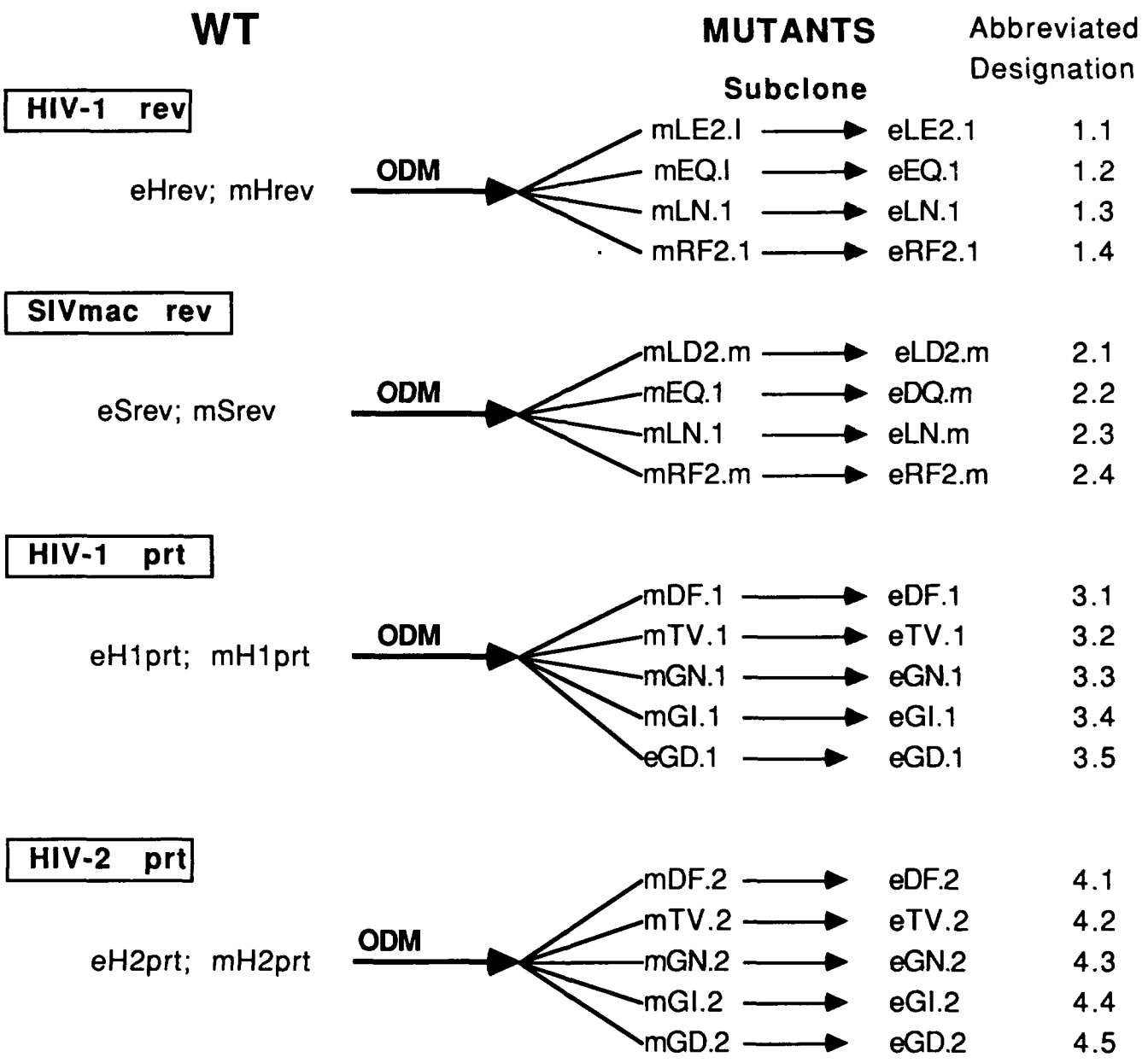
Table ..

PCR PRIMERS

	NAME	LENGTH	SEQUENCE (5'-------3')
HIV-1	PP-3.. 5'	33	ATA TAT ATC TAG ATG GCA GGA AGA AGC GGA GAC
rev	PP-4 .3'	33	GCG CGC GGA GCT CTA TTC TTT AGC TCC TGA CTC
SIV_{mac}	PP-5.. 5'	33	ATA TAT ATC TAG ATG AGC AGT CAC GAA AGA GAA
rev	PP-6.. 3'	32	GCG CGC GGA GCT CAG TCC TGA GGA CTT TTC GA
HIV-1	PP-7.. 5'	36	ATA TAT ATC TAG ATG CCT CAG GTC ACT CTT TGG CAA
prt	PP-8.. 3'	35	GCG CGC GGA GCT CAA AAA TTT AAA GCTG CAA CCA AT
HIV-2	PP-9 5'	36	ATA TAT ATC TAG ATG CCT CAA TTC TCT CTT TGG AAA
prt	PP-10 3'	..35	GCG CGC GGA GCT CAT AAA TTT AAT GAC ATG CCC AA

TABLE 3

CONSTRUCTIONS



NOTE:

e = in pSVL
m = in M13mp19
ODM = oligonucleotide - directed mutagenesis
Hrev = HIV-1 rev
Srev = SIVmac rev
H1prt = HIV-1 protease
H2prt = HIV-2 protease

TABLE 4.

MUTAGENIC OLIGOS

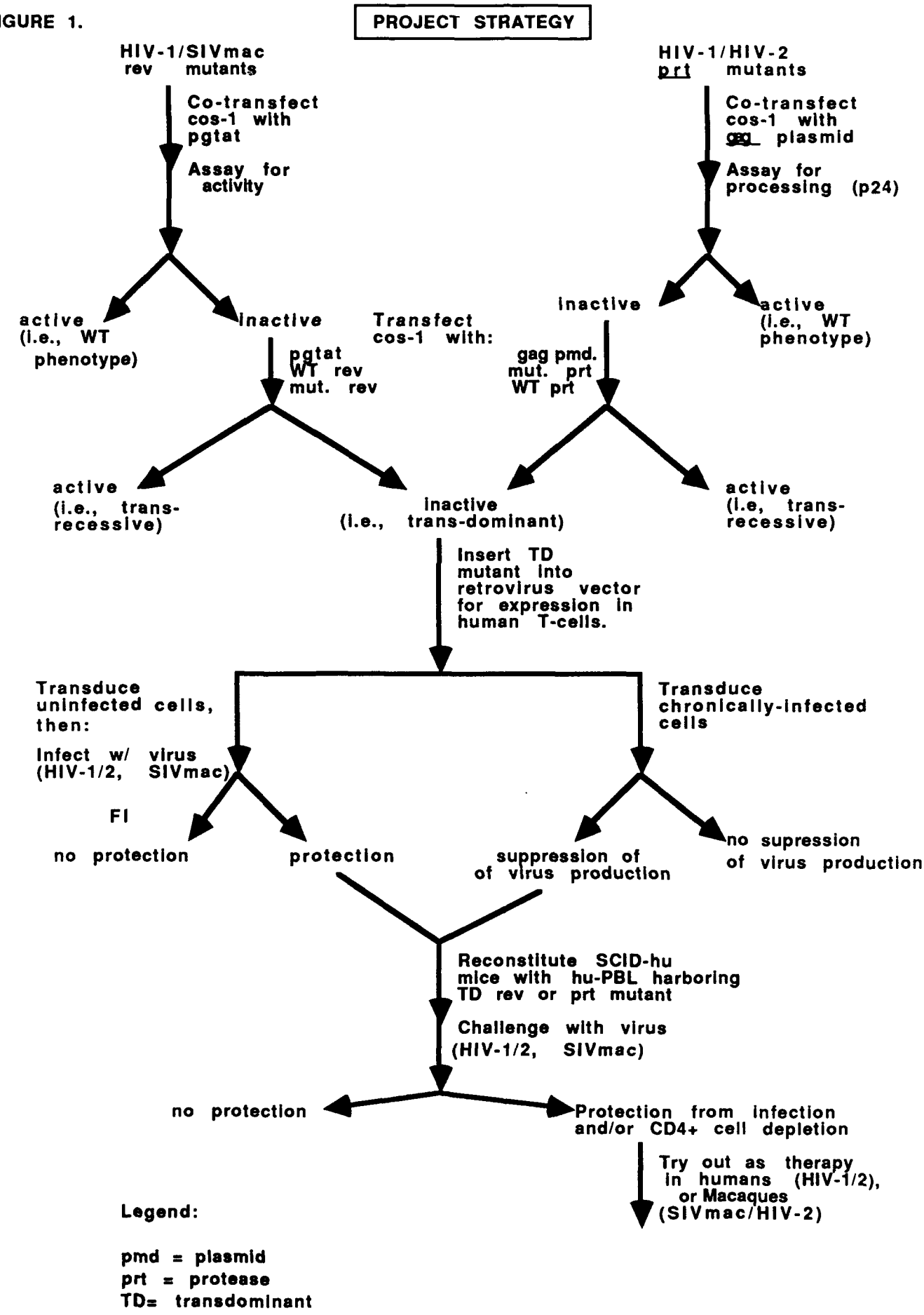
MUTATION DESIGNATION	LENGTH	SEQUENCE (5'------ 3')	MW
HIV-1 rev			
LE2.1	28	pAGA GTA AGT CTA AGC TCC GGT GGT AGC T	9236
EQ.1	19	pGTA AGT CTC TGA AGC GGT G	6293
LN.1	21	pAAG TCT CTC ATT CGG TGG TAG	6947
RF2.1	28	pCTT CTT CTA TTG AAG AAG GCC TGT CGG G	9236
SIV_{mac} rev			
LD2.m	28	pTGA ATA GTC AAA AGG TCA GGC GTA TCA G	9236
DQ.m	21	pAAT AGT CAA CTG AAG AGG CGT	6947
LN.m	21	pAGT CAA GTC ATT AGG CGT ATC	6947
RF2.m	28	pCTC CTT CTT TGG AAG AAC TGG TTG GCA G	9236

TABLE 4. CONTINUED

MUTAGENIC OLIGOS

MUTATION DESIGNATION	LENGTH	SEQUENCE (5'------ 3')	MW
HIV-1 prt			
DF.1	21	TGC TCC TGT AAA TAA TAG AGC	6867
TV.2	21	ATC TGC TCC TAC ATC TAA TAG	6867
GN.1	23	TAT CAT CTG CGT TTG TAT CAT AT	7521
GI.1	21	TTT GA¨ AAA AAT TCC AAT TCC	6867
GD.1	21	CAG ATT TCT GTC AAT TAT GTT	6867
HIV-2 prt			
DF.2	21	AGC CCC TGT GAA TAG TAA AAC	6867
TV.2	21	GTC AGC CCC TAC GTC TAG TAA	6867
GN.2	23	AGT CGT CAG CGT TTG TGT CTA GT	7521
GI.2	21	ATT TAT GAA TAT TCC TAT TCC	6867
GD.2	19	ATA TTT CTG TCA AAA ATG T	6213

FIGURE 1.


PROJECT STRATEGY
HIV-1/SIVmac rev mutants
Co-transfect cos-1 with pgtat
Assay for activity
active (i.e., WT phenotype)
inactive
pgtat WT rev mut. rev
active (i.e., trans-recessive)
HIV-1/HIV-2 prt mutants
Co-transfect cos-1 with gag plasmid
Assay for processing (p24)
inactive
active (i.e., WT phenotype)
Transfect cos-1 with:
gag pmd. mut. prt WT prt
active (i.e, trans-recessive)
inactive (i.e., trans-dominant)
Insert TD mutant into retrovirus vector for expression in human T-cells.
Transduce uninfected cells, then:
Infect w/ virus (HIV-1/2, SIVmac)
FI
no protection
protection
Transduce chronically-infected cells
suppression of of virus production
no supression of virus production
Reconstitute SCID-hu mice with hu-PBL harboring TD rev or prt mutant
Challenge with virus (HIV-1/2, SIVmac)
no protection
Protection from infection and/or CD4+ cell depletion
Try out as therapy in humans (HIV-1/2), or Macaques (SIVmac/HIV-2)
Legend:
pmd = plasmid
prt = protease
TD= transdominant

Cloning Strategy for rev and prt

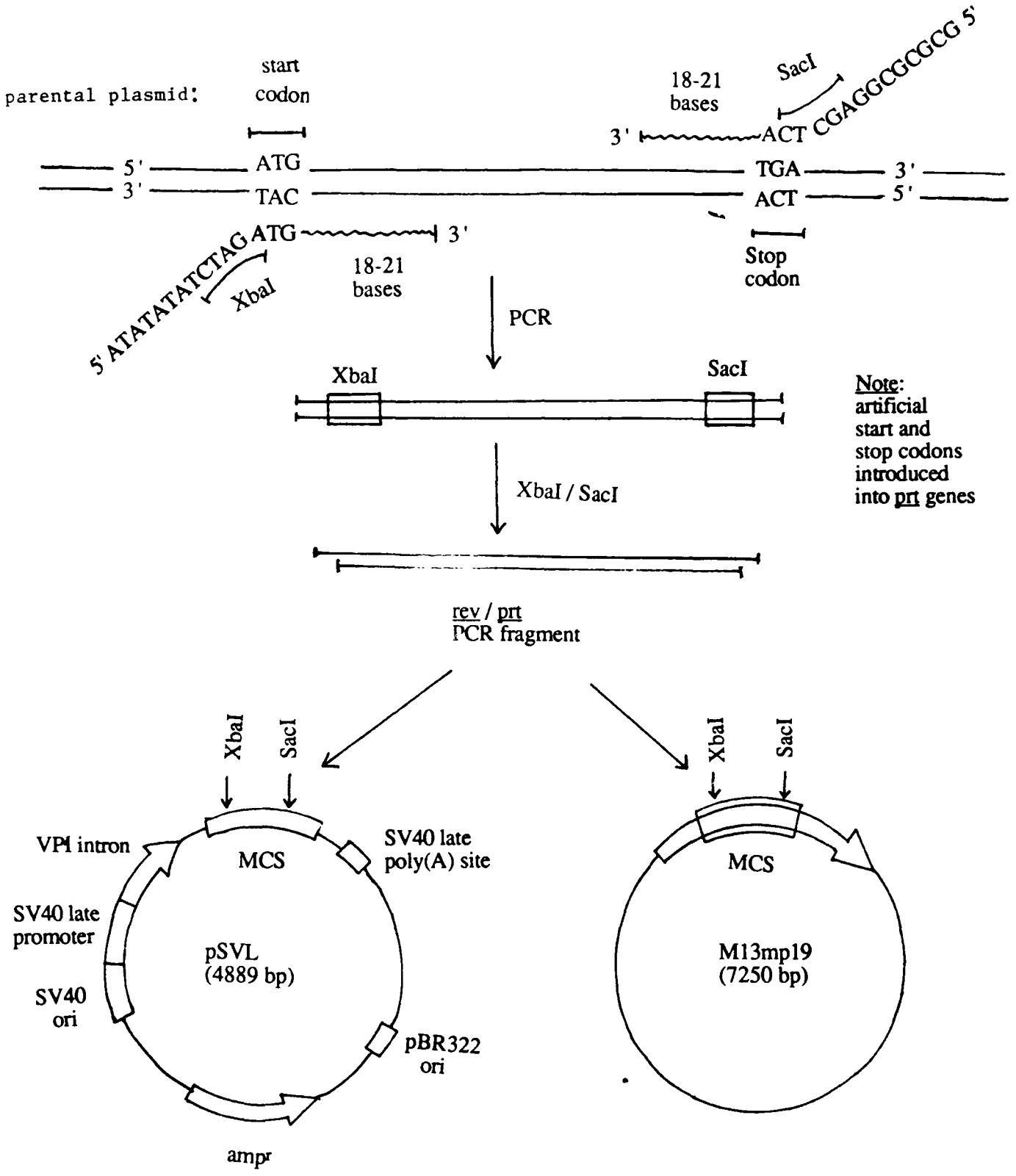


Figure 2

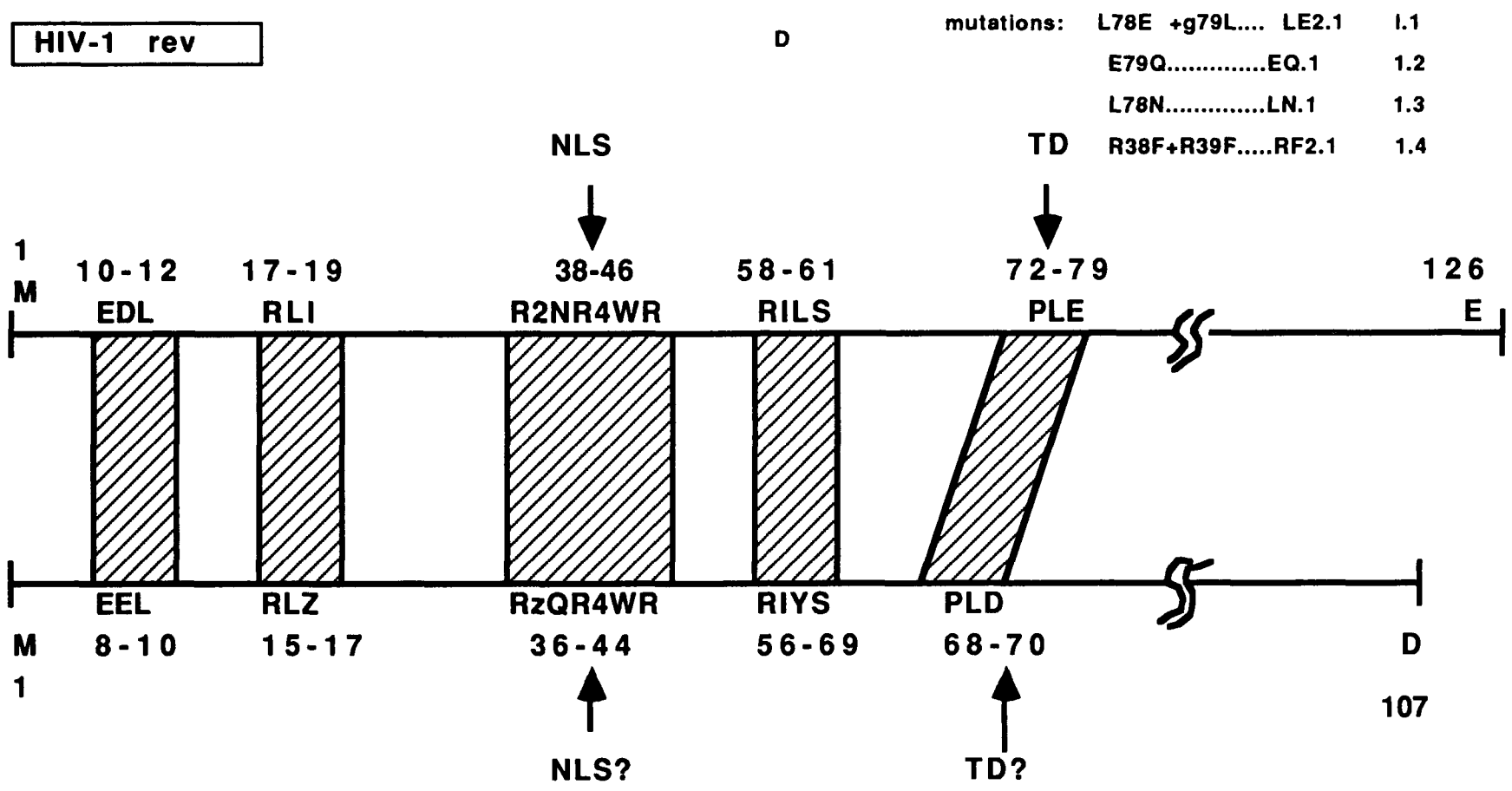

HIV-1 rev
D
mutations: L78E +g79L.... LE2.1 I.1
E79Q.....EQ.1 1.2
L78N.....LN.1 1.3
R38F+R39F.....RF2.1 1.4
NLS
TD
1
M
10-12
EDL
17-19
RLI
38-46
R2NR4WR
58-61
RILS
72-79
PLE
126
E
EEL
RLZ
RzQR4WR
RIYS
PLD
M
1
8-10
15-17
36-44
56-69
68-70
D
107
NLS?
TD?
SIVmac rev
mutations: LgaD + D70L.....LD2.M 2.1
D70Q..... DQ.M 2.2
L69N.....LN.M 2.3
R36F + R37F..... RF2.M 2.4

Fig. 3A

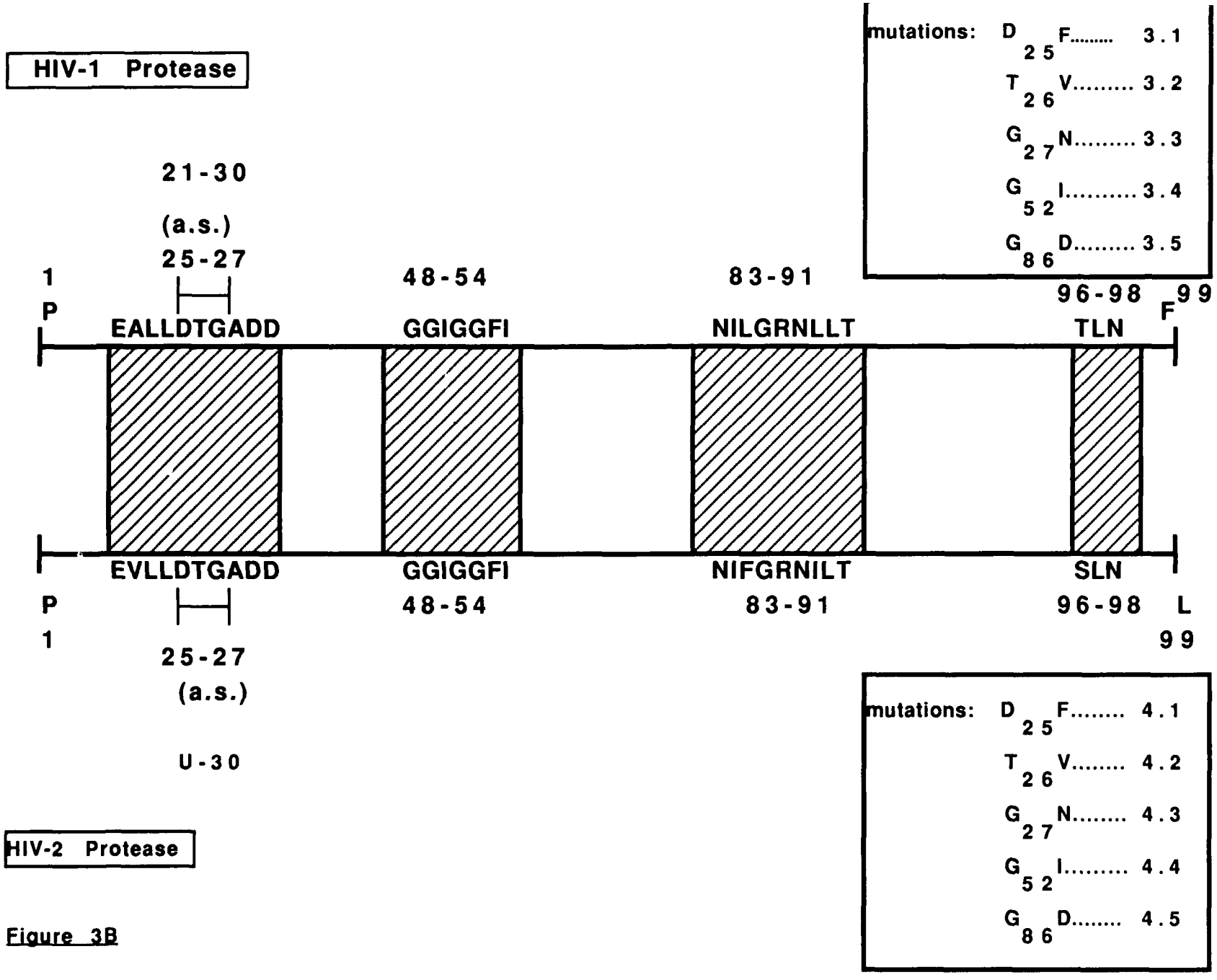

HIV-1 Protease
21-30
(a.s.)
25-27
1
P
EALLDTGADD
48-54
GGIGGFI
83-91
NILGRNLLT
96-98
TLN
F
99
mutations: D25F..... 3.1
T26V..... 3.2
G27N..... 3.3
G52I..... 3.4
G86D..... 3.5
EVLLDTGADD
GGIGGFI
NIFGRNILT
SLN
P
1
25-27
(a.s.)
U-30
48-54
83-91
96-98
L
99
mutations: D25F..... 4.1
T26V..... 4.2
G27N..... 4.3
G52I..... 4.4
G86D..... 4.5
HIV-2 Protease

Figure 3B

Cells: COS-1
Transfection method: Calcium phosphate
Plasmid designation: 1.3=1403 ; 01= 1401 ; 04=1404 ; WT= Wild type Rev
Cloning vector p-SVL

ug of plasmid p-III-AR	1	1	1	1	1	1	1	1	1	1	1	1
ug of plasmid p-Tat	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	.5
ug of plasmid	0	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	2
plasmid designation	1.3	1.3	0.1	0.1	0.4	0.4	WT	WT	WT/1.3	WT/1.3	WT/1.3	

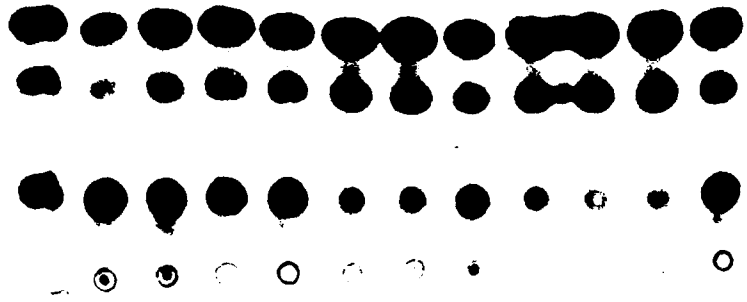


Figure 4A

Cells: HeLa-Rev
Transfection method: Calcium phosphate

ug of plasmid p-Tat	0.5	0.5	0.5	0.5	0.5	0.5
ug of plasmid p-III-AR	1	1	1	1	1	1
ug of plasmid WT-REV	0	1	1	1	1	1
ug of plasmid 1.3	1	1.5	2	3	6	0

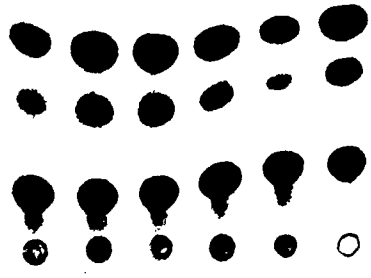


Figure 4B

REFERENCES:

Cullen, B., Hauber, J., Campbell, K., Sodroski, J., Haseltine, W., Rosen, C. 1988. Subcellular localization of the human immunodeficiency virus trans-acting art gene product. *J. Virol.* 62:2498-2501

Feinberg, M.B., Jarrett, R. F., Aldovini, A., Gallo, R.C. and Wong-Staal, F. 1986. HIV regulation involves complex splicing and post-transcriptional events. *Cell* 46:807-817

Felber, B., Derse, D., Athanassopoulos, A., Campbell, M. and Pavlakis, G.N. 1989. Cross-activation of the rex proteins of HTLV-1 and BLV and of the rev protein of HIV-1 and non-reciprocal interactions with their RNA responsive elements. *The New Biol.* 1:318-330.

Malim, M., Bohnlein, S., Hauber, J., Cullen, B., 1989 . Functional dissection of the HIV-1 Rev trans-activator-derivation of a trans-dominant repressor of rev function. *Cell* 58:205 - 204

Malim, M. and Cullen, B. 1991. HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency. *Cell* 65:241-248

Matsukura, M., Zon, G., Shinozuka, K., Robert-Guropff, M., Shimada, T., Wong-Staal, F., Cohen, J. and Broder, S. 1989. *Proc. Nat. Acad. Sci. USA* 86:4244-48

Sambrook, J., Fritsch, E.F., and Maniatas, T., 1987. In: *Molecular Cloning: A Laboratory Manual, 2nd Ed.*, Vol. 2, pp. 1563-1565.

Vaishnav, Y. and Wong-Staal, F. 1991. The Biochemistry of AIDS. *Ann. Rev. Biochem.* 60:577-630.

Wingfield, P.T., Stahl, S.J., Payton, M.A., Greber, D., Venkatesan, S., and Steven, A.C. 1991: HIV-1 rev expressed in recombinant *Escherichia coli*: Purification polymerization and conformational properties: *Biochemistry*, in press.

Wong-Staal, F. 1991. Role of tat and rev in HIV Regulation. In: *HIV Advances in Research and Therapy*. Vol. 1. pp 3-9.